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Characterization of Atrial Natriuretic Peptide Degradation by Cell-Surface Peptidase Activity on Endothelial Cells

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Abstract Atrial natriuretic peptide (ANP) is a fluid-regulating peptide hormone that promotes vasorelaxation, natriuresis, and diuresis. The mechanisms for the release of ANP and for its clearance from the circulation play important roles in modulating its biological effects. Recently, we have reported that the cell surface of an endothelial cell line, CPA47, could degrade ^{125}I-ANP in the presence of EDTA. In this study, we have characterized this degradation of ^{125}I-ANP. The kinetics of ANP degradation by the surface of CPA47 cells were first order, with a K_m of 320 ± 60 nM and V_{max} of 35 ± 14 pmol of ANP degraded/10 min/10^5 cells at pH 7.4. ANP is degraded by the surface of CPA47 cells over a broad pH range from 7.0–8.5. Potato carboxypeptidase inhibitor and bestatin inhibited ^{125}I-ANP degradation, suggesting that this degradative activity on the surface of CPA47 cells has exopeptidase characteristics. The selectivity of CPA47 cell-surface degradation of ANP was demonstrated when ^{125}I-ANP degradation was inhibited in the presence of neuropeptide Y and angiotensin I and II but not bradykinin, bombesin, endothelin-1, or substance P. The C-terminal amino acids phe^{26} and tyr^{28} were deduced to be important for ANP interaction with the cell-surface peptidase(s) based on comparison of the IC_{50} of various ANP analogues and other natriuretic peptides for the inhibition of ANP degradation. These data suggest that a newly characterized divalent cation-independent exopeptidase(s) that selectively recognizes ANP and some other vasoactive peptides exists on the surface of endothelial cells.

Key words: proteolysis, ANP, CPA47, vasoactive peptides, clearance

Atrial natriuretic peptide (ANP) is a fluid-regulating peptide hormone that promotes vasorelaxation, natriuresis, and diuresis and inhibits pressor and volume-conserving hormones [1]. ANP, a 28 amino acid peptide, is a C-terminal cleavage product of a 126–amino acid prohormone that is secreted as a result of elevated intracardiac pressure and increased tension in the atrial wall [1]. This peptide contains a 17-member ring created by an intradisulfide bond that is necessary for its biologic activity.

The mechanisms for the release and clearance of ANP from the circulation play important roles in modulating its biological effects. ANP is removed rapidly from the circulation in both rats and humans [2,3]. ANP's rapid degradation and clearance from the bloodstream has been postulated to occur through at least two different mechanisms: 1) ligand binding to nonguanylate cyclase-linked ANP receptors (C-ANPR) [4–7] and 2) enzymatic degradation by peptidases such as endopeptidase 3.4.24.11 (NEP 24.11) and kallikrein [4,5,8–10].

Recently, two independent investigators examined the effects of an endopeptidase 24.11 inhibitor and a truncated analog of ANP (amino acids 4–23) on ANP's biological activity, clearance, and degradation in vivo [4,5]. Seymour and coworkers [4] reported that in normotensive rats, plasma ANP concentration and its biological effects are not significantly increased when NEP 24.11 inhibitors and ANP4–23 are infused, suggesting that enzymes other than NEP 24.11 are involved in the degradation of ANP in normal rats. Chiu and coworkers [5] studied the pharmacokinetics of ANP in rats. They reported that ANP4–23 alone could significantly inhibit ANP clearance, and endopeptidase inhibitor SCH 39370 could significantly inhibit ANP degrada-

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tion within the first 10 min after infusion. However, clearance and degradation could be only partially inhibited by the combination of both drugs. Furthermore, when the kidneys (the major source of NEP 24.11 and other endopeptidases) were removed from rats, ANP degradation was as effective during the first 10 min of infusion with ANP4–23 and radiolabeled ANP as in non-nephrectomized rats. Thus, it is likely that enzymes other than kidney endopeptidases are involved in the initial degradation of ANP in vivo.

One proteolytic activity that could be involved in ANP degradation is a divalent cation-independent cell-surface proteolytic activity recently reported on a cell line derived from bovine pulmonary artery, CPA47 [11]. This proteolytic activity could be important in the regulation of ANP's biological action since endothelial cells lining blood vessels would be the first cell type to interact with ANP. Cell-surface peptidases have been reported on endothelial cells and are believed to control the biological activity of various neuropeptides [12,13]. Thus, the metabolism of ANP by a cell-surface peptidase(s) on endothelial cells could play an important role in the regulation of ANP's biological activity. In this paper, we have characterized the CPA47 cell-surface peptidase(s) responsible for the degradation of ANP in vitro.

METHODS

Materials

Human ANP was purchased from Peninsula Laboratories (Belmont, CA). Fetal calf serum and bovine calf serum were obtained from Hyclone (Logan, UT). Human ^{125}I-ANP with a specific activity of 2,000 Ci/mmole was purchased from Amersham (Arlington Heights, IL). Burdick and Jackson's HPLC solvents were acquired from Baxter Scientific (McGraw Park, IL). Human ANP, rat brain natriuretic peptide (BNP), rat atriopeptin I, and rat atriopeptin II were obtained from Peninsula Laboratories (Belmont, CA). Rat C-type natriuretic peptide (CNP) was purchased from Bachem (Torrance, CA). All other chemicals and peptides were purchased from Sigma (St. Louis, MO).

Cell Culture

CPA47 cells (ATCC CRT 1733) were obtained from and cultured according to American Type Culture Collection (Rockville, MD) with the exception that cells were trypsinized when passaged and the cells were cultured in 5% CO_2. Cells were not used after passage 40 (i.e., 13 passages of ATCC obtained cells). Cells were seeded at a density of 1.7–2.7 $\times$ 10^4 cells/cm^2 in 12-well plates, were 90–100% confluent when the assays were performed, and were used within 7 days after subculturing.

Kinetics of ANP Binding at 4°C

CPA47 cells were washed twice in binding medium (BM; 1:1 ratio of F-12 (Sigma #N-6760) and Minimal Essential Medium (Sigma #M-0643) containing 6 g/l Hepes, and 4 g/l Tricine, pH 7.4, with 50 μg/ml of bovine serum albumin [14] with 2 mM EDTA at 4°C. Then 340 μl of BM containing 2 mM EDTA (BME) was added per well and equilibrated at 4°C for 10 min. The cells were incubated at 4°C with 35–40 pM radiolabeled ANP in the absence (total binding) or presence (nonspecific binding) of nonradiolabeled ANP (100 nM) in 0.4 ml of BME for increasing lengths of time. At each time point, the solution was aspirated, and the cells were washed twice in BME and solubilized in 1 N NaOH. A fraction of the solubilized cells was used to determine the radioactivity in each well. Specific binding was determined by subtracting the nonspecific binding from the total binding at each time point.

Equilibrium Binding of ANP to CPA47 Cells at 4°C

Equilibrium binding studies were performed as described by Whitson and coworkers [14]. Briefly, CPA47 cells cultured in 12-well plates were washed twice in BM or BME. The cells were incubated at 4°C with 35–40 pM radiolabeled ANP containing increasing amounts of nonradiolabeled ANP (0–100 nM) in 0.4 ml of BM or BME. The cells were then incubated at 4°C for 1 h, and the cells were washed twice in the appropriate medium. The nonspecific binding (i.e., ^{125}I-ANP bound to cells in the presence of 100 nM nonradiolabeled ANP) accounted for less than 20% of total ^{125}I-ANP binding.

Kinetics of ^{125}I-ANP Degradation by CPA47 Cells

CPA47 cells were washed twice with BM followed by one wash in BME. Next, 340 μl of BME was incubated with cells for 5–10 min at 4°C to equilibrate the cells. The cells were then incubated with 100–600 nM nonradiolabeled ANP

containing radiolabeled ANP (70 pM) for 10 min at 4°C. A sample of each concentration of ANP added to the cells was analyzed for total radioactivity. After incubation, the medium was removed and centrifuged at 13,000*g* for 2 min to remove any large debris. The supernatant was removed, a fraction was used to determine the recovered amount of ANP, and the remaining supernatant (340 μl) was frozen at −70°C until a Sep-Pak assay was performed to quantitate ANP degradation.

Effect of pH on Degradation of CPA47 Cells

CPA47 cells cultured in 12-well plates were washed twice with BME containing 25 mM Tris-HCl, 25 mM Mes, and 25 mM Pipes at different pH (5.0–9.0), and incubated with 340 μl of BME at the appropriate pH for 5–10 min at 4°C. The cells were then incubated with radiolabeled ANP (60 pM) for 10 min at 4°C. The medium was removed and centrifuged at 13,000*g* for 2 min to remove any large debris. Mock-treated wells were used to determine the pH of the medium during the incubation of CPA47 cells with ANP. The supernatant was removed, and a fraction was used to determine the amount of ANP recovered. The remaining supernatant (340 μl) was frozen at −70°C until a Sep-Pak assay was performed to quantitate ANP degradation.

Sep-Pak Assay to Quantitate ANP Degradation

^{125}I-ANP degradation was determined by Sep-Pak fractionation according to Morel et al. [31] with minor modifications. Briefly, samples were thawed in the presence of 0.4 N HCl and ANP was adsorbed onto methanol- and water-washed C_{18} Sep-Pak cartridges (Waters, Milford, MA). Degraded ANP was eluted with 20% acetonitrile in 0.1% TFA. The radioactivity remaining on the Sep-Pak cartridge represented the intact ANP. The degradation of ANP was quantitated by subtracting the amount of degraded radiolabeled ANP in BM or BME alone (1–7%) from the observed ANP degradation. The percent degradation of ANP was determined by the equation [100 × (degraded ANP/{intact + degraded ANP})]. To quantitate the ANP degraded, the percent degradation of ANP was multiplied by the radioactivity added to each well and divided by the specific activity of ANP and by the number of cells per well. Units of ANP degradation are listed as pmol of ANP degraded/10 min/10^5 cells. The sensitivity of this assay was modulated two ways: 1) by increasing the length of time for the assay, creating a higher percentage of degradation products and 2) by increasing radiolabeled ANP concentrations. Initial degradation rates for radiolabeled ANP (i.e., kinetics of ANP degradation and IC_{50} for ANP degradation by various peptides) required a shorter incubation time to limit the amount of degradation products. Hence, the radiolabeled ANP concentrations were increased for these assays. When the accumulation of degradation products was not as critical, ANP degradation was determined with 1 h incubations to increase the degradation products created by CPA47 cell surface proteolytic activity.

RESULTS

HPLC Profile of ^{125}I-ANP Degradation at 4°C Separated by Sep-Pak Procedure

Radiolabeled ANP was incubated with CPA47 cells in BM with 2 mM EDTA at 4°C for 1 h. This temperature was selected to inhibit receptor-mediated and fluid-phase endocytosis [16], and EDTA was included in the BM to inhibit ANP degradation by a cell-derived factor released into the medium [11]. Hence, only surface degradation was observed under these conditions. After a 1 h incubation, the medium samples were analyzed by HPLC to separate the degradation products of ^{125}I-ANP from intact ^{125}I-ANP. Intact ANP eluted between 30–33 min on a C_{18} HPLC column (Fig. 1A). However, three new peaks of radioactivity were observed after incubation with CPA47 cells (Fig. 1B). The peaks at 15 min and 17 min corresponded to ^{125}I-tyr^{28} and arg^{27}-^{125}I-tyr^{28} respectively, based on previously published data using C_{18} HPLC columns with similar gradients [6,17,18]. Since this degradation occurred at 4°C, proteolytic activity on the surface of CPA47 cells is suggested.

^{125}I-ANP degraded by CPA47 cells was also analyzed, in a separate experiment, by a rapid assay using C_{18} Sep-Pak cartridges. Partially degraded radiolabeled ANP was adsorbed onto the Sep-Pak cartridge and the degraded ANP represented in peaks 15 and 17 were eluted from the Sep-Pak cartridge with 6 ml of 20% acetonitrile in 0.1% TFA (Fig. 1C). The remaining radioactivity on the Sep-Pak cartridge was eluted with 60% acetonitrile in 0.1% TFA; it eluted at the same position as intact ANP (Fig. 1D). It should also be noted that the small peak observed at 28 min may not be completely separated from the intact ANP using the Sep-Pak assay technique. Thus, the percent degradation

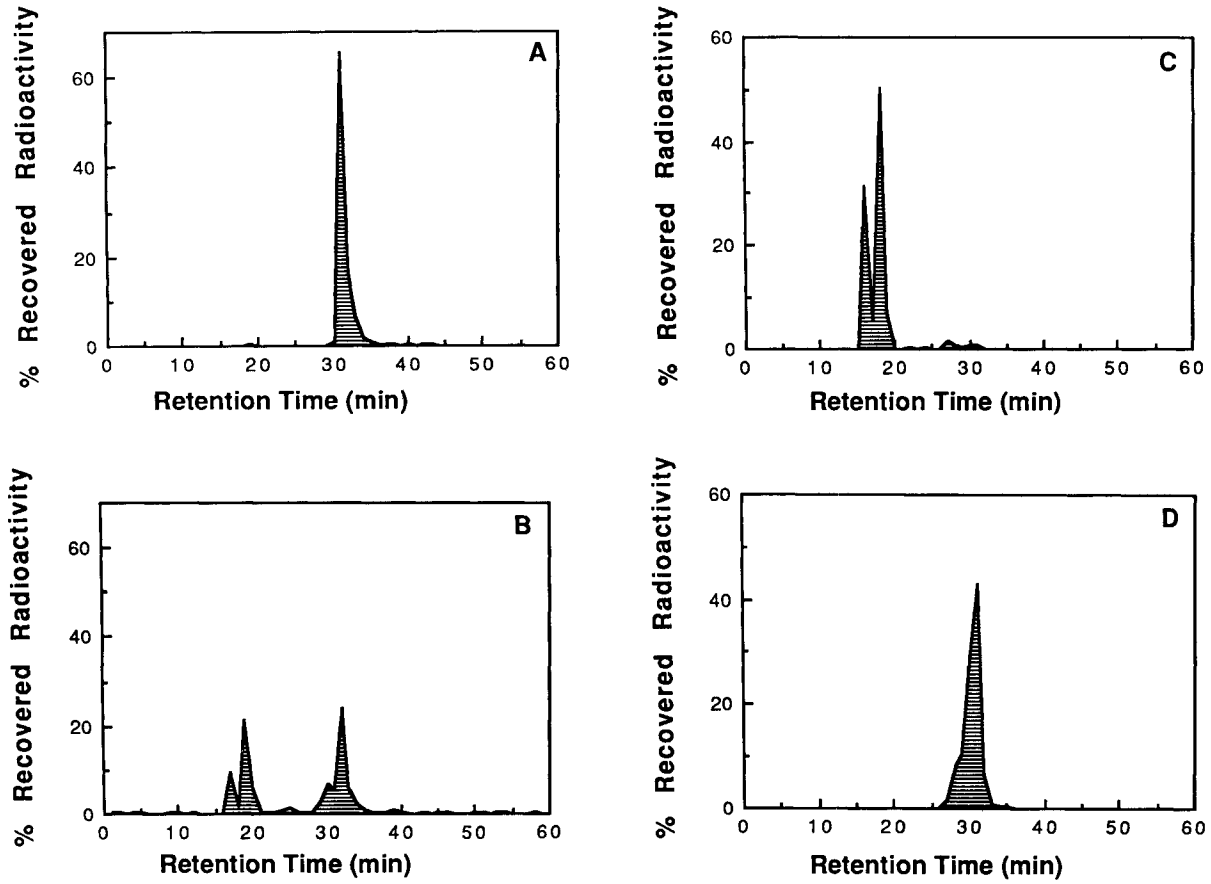

Fig. 1. HPLC analysis of ^{125}I-ANP degradation products by Sep-Pak separation. CPA47 cells were preincubated with BME (for A,B) or BM (for C,D) for 5–10 min. After equilibration, the cells were incubated with 50 pM ^{125}I-ANP for 1 h at 4°C, and the degraded ^{125}I-ANP in the medium was analyzed by HPLC as previously reported [15]. The radioactivity in each fraction was determined by a Packard Auto-Gamma counter. The percent recovered radioactivity was calculated by dividing the radioactivity in each fraction by the radioactivity recovered in all fractions. **A:** Control: ^{125}I-ANP incubated in BME for 1 h at 4°C. **B:** Cells incubated in BME with ^{125}I-ANP for 1 h at 4°C. **C:** ^{125}I-ANP eluted from Sep-Pak by 20% acetonitrile/0.1% TFA. **D:** ^{125}I-ANP eluted from Sep-Pak by 60% acetonitrile/0.1% TFA after the degraded ANP was eluted by 20% acetonitrile/0.1% TFA.

observed using the Sep-Pak assay may underestimate the actual degradation of radiolabeled ANP. However, the percent degradation of ^{125}I-ANP as determined by HPLC was not significantly greater than the degradation determined by Sep-Pak assay ($P > 0.05$; n = 4). Other investigators have also used this technique for quickly determining the amount of degraded ANP [31].

One shortcoming of using ^{125}I-ANP for characterizing ANP degradation is that the ^{125}I-tyr is the C-terminal amino acid. Once the C-terminal amino acid is removed, the degradation of the remaining ANP molecule cannot be followed. Thus, this technique is more sensitive to C-terminal degradation of ANP. Even with this limitation, CPA47 cells could generate radiolabeled ANP fragments that could be separated using either HPLC or Sep-Pak assay. Degradation of nonradiolabeled ANP by CPA47 cells was attempted at 4°C. However, different binding mediums either interfered with the detection of ANP fragments at OD_{214} or caused cells to detach from the plate. Thus, radiolabeled ANP was used to characterize ANP degradation by endothelial cells.

Effect of pH on Degradation of ^{125}I-ANP

We also investigated the effects of pH on ^{125}I-ANP degradation. The pH range was limited to maintain viable cells for the assay, and the ^{125}I-ANP degradation by the surface of CPA47 cells had a broad pH range (Fig. 2). The optimum pH for the ANP degradation was pH 7.5 in two of three experiments. Substantial ANP degradation occurred within the pH range from 7.0–9.0. However, ANP degradation was greatly reduced

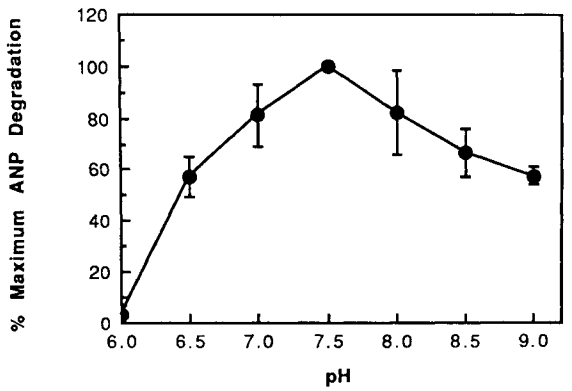

Fig. 2. Effect of pH on the surface degradation of ANP by CPA47 cells. CPA47 cells (130,000–210,000 cells/well; passage 9) were preincubated at 4°C in BME at the indicated pH. After equilibration, the cells were then incubated with ^{125}I-ANP (60 pM) ANP for 10 min at 4°C, and the degraded ANP in the medium was determined by Sep-Pak assay as described in Methods. Each point represents the mean of three independent experiments; error bars indicate the standard error of the mean (SE). ANP degradation at each pH was standardized to the degradation obtained at pH 7.5 for each experiment. The average degradation for the three independent experiments at pH 7.5 was 5.4% ± 0.9% ^{125}I-ANP degradation/10 min/10^5 cells.

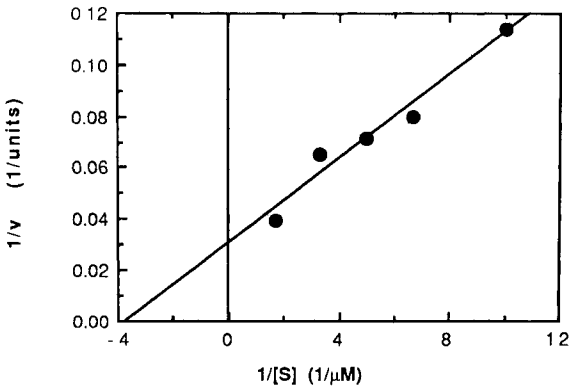

Fig. 3. Kinetics of ^{125}I-ANP degradation. The kinetics of radiolabeled ANP degradation were performed as described in Methods with a constant amount of radiolabeled ANP and increasing concentrations of nonradiolabeled ANP. The degraded radiolabeled ANP was analyzed by Sep-Pak assay as described in Methods. The kinetics of ANP degradation were analyzed by Lineweaver-Burke plot. Linear regression analysis was performed to determine the x-intercept ($-1/K_m$) and y-intercept ($1/V_{max}$) and the correlation coefficients of least-squares linear regression were > 0.95.

at acidic pH (6.0). A pH of 7.4 was selected for subsequent characterization since this value approximates the pH of blood.

Kinetics of ^{125}I-ANP Degradation

The kinetics of ANP degradation were examined by increasing the concentration of nonradiolabeled ANP in the presence of a constant amount of radiolabeled ANP following a 10 min incubation at 4°C in the presence of 2 mM EDTA. In initial experiments, we observed minimal changes in the degradation of radiolabeled ANP in the presence of 100 nM nonradiolabeled ANP. Therefore, we used a concentration range of 100 nM to 600 mM nonradiolabeled ANP in later experiments. Under these conditions, the enzyme kinetics are first order and the K_m and V_{max} were estimated from a linear regression of a Lineweaver-Burke plot (Fig. 3). When five independent experiments were analyzed, the apparent K_m was 320 ± 30 nM and V_{max} was 35 ± 6 pmol of ANP degraded/10 min/10^5 cells at pH 7.4.

Effect of Various Protease Inhibitors on ^{125}I-ANP Degradation

Peptidases can be classified based upon their sensitivities to various protease inhibitors [19]. We quantified ANP degradation after CPA47 cells were treated with a variety of protease inhibitors. One peptidase on the CPA47 cell surface could be classified as a carboxypeptidase, since approximately 90% of the ANP degradation was inhibited by potato carboxypeptidase inhibitor (Table I). Other protease inhibitors demonstrated limited or no effects on ANP degradation (Table I). Serine, acid, and thiol protease inhibitors did not inhibit ANP degradation (i.e., aprotinin, leupeptin, E-64, pepstatin, and phenyl methyl sulfonyl fluoride [PMSF]). Bestatin, an aminopeptidase inhibitor, and phosphoramidon, a metalloprotease inhibitor, had minor effects on ANP degradation (40% and 31% inhibition, respectively).

Since potato carboxypeptidase effectively prevented radiolabeled ANP degradation, the IC_{50} (concentration that inhibits ANP degradation 50%) was determined by least-squares linear regression on a Dixon plot. The IC_{50} for potato carboxypeptidase inhibition of two independent experiments using increasing concentrations of potato carboxypeptidase inhibitor was 2.6 μM ± 0.5 μM.

Specificity of ^{125}I-ANP Degradation

Various biologically active peptides were incubated with radiolabeled ANP at 4°C to determine whether peptides other than ANP could inhibit degradation of radiolabeled ANP by the surface of CPA47 cells. An initial concentration

TABLE I. Effect of Various Protease Inhibitors on Cell-Surface Degradation of ANP*

Protease inhibitor	Concentration (μg/ml)	Inhibition (%)
Potato carboxypeptidase inhibitor	420	88 ± 3
Bestatin	40	40 ± 6
Phosphoramidon	370	31 ± 6
Phenyl methyl sulfonyl fluoride (PMSF)	230	5 ± 10
E-64[a]	1,000	−2 ± 21
Aprotinin	10	−4 ± 17
Leupeptin	1	−13 ± 13
Pepstatin A	1	−13 ± 9

*CPA47 cells (100,000–120,000 cells/well) were equilibrated at 4°C in BME containing various protease inhibitors for 5–10 min. The cells were then incubated at 4°C for 1 h in the presence of 110 pM ^{125}I-ANP, and the ANP degraded in the medium was determined by Sep-Pak assay as described in Methods. The percent inhibition of ANP degradation was calculated as (1 − [radiolabeled ANP degradation in the presence of peptide inhibitor/ANP degradation in the absence of peptidase inhibitor]) × 100. ANP degradation in the absence of protease inhibitors per 10^5 cells was 3.2% ± 0.8% (n = 3). The concentrations of protease inhibitors selected are equal to or greater than concentrations used to classify other ANP enzymes [20, 21]. The percent inhibition of ^{125}I-ANP degradation is the mean of three independent experiments ± SE (standard error of the mean).

[a]E-64, trans-epoxysuccinyl-l-leucylamido-(4-guanidino)-butane.

of 3 μM (tenfold greater than the apparent K_m for ^{125}I-ANP) was selected to maximize the inhibition of the cell-surface peptidase(s) by nonradiolabeled ANP. Some of the vasoactive peptides partially inhibited ANP degradation. Neuropeptide Y was 94% as effective as nonradiolabeled ANP at inhibiting ^{125}I-ANP degradation (Table II). Angiotensin I and II inhibited ANP degradation 60–70% as effectively as nonradiolabeled ANP (Table II). Other peptides such as endothelin-1, bombesin, bradykinin, and substance P did not greatly affect radiolabeled ANP degradation (< 19% as effective as nonradiolabeled ANP without protease inhibitors). These peptides were also tested in the presence of protease inhibitors since their inability to inhibit radiolabeled ANP degradation could be due to the degradation of these peptides by other enzymes on the cell surface. Even in the presence of protease inhibitors, PMSF, leupeptin, and pepstatin, inhibition of radiolabeled ANP degradation was only slightly increased by endothelin-1 (22% ± 5.4; n = 2) and bradykinin (32% ± 7.3; n = 2) as compared to data presented in Table II.

TABLE II. Effect of Various Peptides on Degradation of Radiolabeled ANP by Surface Peptidase(s) Activity of CPA47 Cells*

Vasoactive peptide	Inhibition (3 μM) (%)	IC_{50} (μM)
ANP	88 ± 1	0.47 ± 0.09
Neuropeptide Y	81 ± 2	0.48 ± 0.18
Angiotensin II	66 ± 2	1.37 ± 0.19
Angiotensin I	48 ± 6	1.70 ± 0.12
Substance P	17 ± 7	> 3 μM
Bradykinin	13 ± 3	> 3 μM
Endothelin-1	−3 ± 3	> 3 μM
Bombesin	− 5± 8	> 3 μM

*CPA47 cells (80,000–150,000 cells/well) were equilibrated at 4°C in BME and incubated at 4°C for 1 h with radiolabeled ANP (60 pM) in the presence or absence of various peptides (3 μM). The degraded ANP in the medium was determined by Sep-Pak assay as described in Methods. The percent inhibition of ANP degradation was calculated as (1 − [radiolabeled ANP degradation in the presence of peptide/ANP degradation in the absence of peptide]) × 100, and is presented as the mean and SE of three independent experiments. The degradation of radiolabeled ANP by CPA47 cells in the absence of peptides was 3.4% ± 1.1% (n = 3) per 10^5 cells per 10 min. For the determination of the IC_{50} of the various vasoactive peptides, CPA47 cells (130,000–210,000 cells/well) were equilibrated at 4°C in BME containing 230 μg/ml PMSF, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A, and incubated at 4°C for 10 min with 60 pM radiolabeled ANP. The degradation of ANP was determined as described in Methods. The IC_{50} was determined as the negative x-intercept of a least-squares linear regression on a Dixon plot. Numbers are given as the mean and SE of percent inhibition and IC_{50} from two independent experiments. The ANP degradation in the absence of ANP analogues was 11.9% ANP degradation/10^5 cells/10 min (n = 2). The IC_{50} for nonradiolabeled ANP was obtained from the experiments performed in Table III and is included for comparison. The correlation coefficients for all linear regressions were greater than 0.87.

The IC_{50} of neuropeptide Y and angiotensins I and II were investigated since these peptides had > 50% inhibitory effect on radiolabeled ANP degradation by CPA47 cells at 3 μM. Neuropeptide Y was the most effective inhibitor of ANP degradation with an IC_{50} of 480 nM (Table II). Angiotensin I and II had similar IC_{50}: 1.7 μM for angiotensin I and 1.4 μM for angiotensin II, respectively (Table II). The IC_{50} of bombesin, bradykinin, substance P, and endothelin-1 are greater than 3 μM since these peptides at this concentration inhibited radiolabeled ANP degradation less than 50%. Thus, some vasoactive peptides were able to block radiolabeled ANP degradation.

Other experiments were performed to determine the specificity of the cell-surface peptidase(s) using ANP analogues. ANP analogues

TABLE III. Effect of ANP Analogues on Degradation of Radiolabeled ANP by a Surface Peptidase(s) Activity of CPA47 Cells*

ANP peptide analogue	Inhibition (3 μM) (%)	IC_{50} (nM)
Atriopeptin III	88 ± 3	270 ± 100
ANP	91 ± 3	470 ± 90
Atriopeptin II	88 ± 2	520 ± 50[a]
Brain natriuretic peptide	65 ± 4	650 ± 150[a]
Atriopeptin I	73 ± 3	840 ± 100[a b c]
C-type natriuretic peptide	72 ± 3	970 ± 120[a b c]

*CPA47 cells (70,000–120,000 cells/well) were equilibrated at 4°C in BME and incubated at 4°C for 1 h with radiolabeled ANP (60 pM) in the presence or absence of various peptides (3 μM) The degraded ANP in the medium was determined by Sep-Pak assay as described in Methods The percent inhibition of ANP degradation was calculated as (1 − [radiolabled ANP degradation in the presence of peptide/ANP degradation in the absence of peptide]) × 100 For the determination of IC_{50} of the ANP analogues, CPA47 cells (65,000–90,000) were equilibrated at 4°C in BME and incubated at 4°C for 10 min with radiolabeled ANP (80 pM) and nonradiolabeled 10 nM ANP containing increasing concentrations of various ANP analogues The 10 nM nonradiolabeled ANP was included in this experiment to prevent the interaction of ANP analogues with the ANP receptors The degradation of ANP was determined as described in Methods The IC_{50} was determined as the negative x-intercept of a least-squares linear regression on a Dixon plot Numbers are given as the mean and SE of percent inhibition and IC_{50} from three independent experiments The ANP degradation in the absence of ANP analogues was 11% ± 1% ANP degradation/10^5 cells/10 min (n = 3) A one-tailed Student t-test was performed using all combinations of ANP and its analogues The correlation coefficients for all linear regressions were greater than 0 92 with the exception of one line that had a correlation coefficient of 0 82

[a]K_i greater than atriopeptin III ($P < 0.05$)

[b]K_i greater than atriopeptin II ($P < 0.05$)

[c]K_i greater than ANP ($P < 0.025$)

such as atriopeptin II (ANP 5–27) and atriopeptin III (ANP 5–28) were nearly as effective at inhibiting radiolabeled ANP degradation as was nonradiolabeled ANP (Table III). However, other ANP analogues such as atriopeptin I (ANP 5–25), rat BNP-32, and C-type natriuretic peptide were only ~70% as effective as nonradiolabeled ANP at inhibiting radiolabeled ANP degradation by CPA47 cells (Table III). These data demonstrate a differential selectivity of the cell-surface peptidase(s) for some ANP analogues.

To further characterize the interaction of ANP analogues with the cell-surface peptidase(s), increasing concentrations of these peptides were added to CPA47 cells containing 10 nM ANP. The apparent IC_{50} for the ANP analogues was analyzed by a Dixon plot. The IC_{50} of atriopeptin I, II, and III were significantly different from each other, with the IC_{50} of atriopeptin III (5–28) < atriopeptin II (5–27) < atriopeptin I (5–25) (Table III). Furthermore, CNP and atriopeptin I had significantly higher IC_{50} than ANP (Table III; $P < 0.05$).

DISCUSSION

In a previous report, we observed substantial degradation of ANP by proteolytic activity located on the surface of CPA47 cells [11]. This activity was shown to be insensitive to EDTA [11]. In this paper, we characterized this surface proteolytic activity further by assessing the selectivity of the process, the pH optimum, and the recognition sites on ANP necessary for degradation. The degradation of ^{125}I-ANP by the cell-surface proteolytic activity on CPA47 cells demonstrated first-order kinetics (i.e., linear Lineweaver-Burke plot). Furthermore, the degradation of ^{125}I-ANP by the CPA47 cell surface demonstrated specificity for some vasoactive peptides and was inhibited by carboxypeptidase inhibitor. Together, these data suggest the presence of a carboxypeptidase-like activity that is divalent cation-independent and located on the surface of CPA47 cells.

The existence of peptidase activity within the ANP receptor itself is unlikely since the K_m of the peptidase is not similar to K_d of ANP receptors on these cells. We observed that the K_d of ANP binding to these cells was 220 ± 40 pM at 4°C in BM (n = 3), whereas the K_m for ANP degradation by the cell-surface protease was three orders of magnitude greater (~300 nM). Since these values are so different, it is unlikely that the ANP receptors on the CPA47 cells are responsible for the proteolytic activity that we have characterized. However, it is possible that two distinct ANP binding sites exist on the receptors, one for ANP binding and one for enzymatic degradation.

We examined selectivity of this cell-surface peptidase activity using various peptides. Peptides that reportedly cause vasoconstriction, such as neuropeptide Y and angiotensin I and II [22,23], were the most effective at inhibiting ANP degradation. Peptide vasodilators, such as bradykinin and substance P [22,23], were not as effective. However, not all peptides that promote vasoconstriction (e.g., endothelin-1 [24]) were effective at inhibiting radiolabeled ANP degradation.

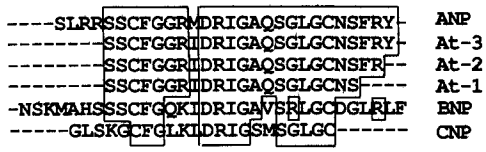

Fig. 4. Amino acid sequence of ANP and related peptides. ANP and related peptides are compared using the one-letter code for each amino acid. The boxed areas are amino acids identical in sequence to those of human ANP.

The structural requirements of vasoactive peptides necessary for recognition by the peptidase on the surface of CPA47 cells are unclear. Little sequence similarity exists among ANP, neuropeptide Y, and angiotensin I and II. However, these data suggest that an intact 17-member ring in ANP is not essential for interaction with the peptidase(s), because neuropeptide Y and angiotensin I and II do not have a disulfide bond. In addition, angiotensin I and II have fewer than 17 amino acids.

Although the structurally important regions of other vasoactive peptides are unclear, data generated using ANP analogues suggest that the C-terminal region of the ANP molecule is involved in the ability of ANP analogues to inhibit radiolabeled ANP degradation (Fig. 4; Table 3). Atriopeptin III (5–28) was as effective at inhibiting ANP degradation by CPA47 cells as ANP, suggesting that the N-terminal portion of ANP is not involved in ANP's recognition by the cell-surface peptidase(s). However, the deletion of the carboxy terminal amino acid tyr^{28} (i.e., atriopeptin II) increased the IC_{50} from 270 nM ± 170 nM (atriopeptin III) to 520 nM ± 90 nM (atriopeptin II) ($P < 0.05$), suggesting that the C-terminal tyr^{28} may be involved in the recognition of ANP by the cell-surface peptidase(s). Furthermore, the deletion of amino acids 26 and 27 (i.e., atriopeptin I) increased the IC_{50} to 840 nM ± 180 nM, which differs significantly from that of atriopeptin II ($P < 0.05$). These data suggest that these three C-terminal amino acids are involved in the recognition of ANP by the cell-surface peptidase(s). This conclusion is consistent with the observation that CNP, which lacks the five C-terminal amino acids, inhibited ANP degradation similarly to atriopeptin I. BNP has leucines at position 26 and 28 instead of phe^{26} and tyr^{28}, and also has a lower IC_{50} than ANP, atriopeptin II, or atriopeptin III. Thus, these data indicate that the C-terminal amino acids phe^{26} and tyr^{28} are involved in ANP recognition by the cell-surface peptidase(s). This conclusion is consistent with the classification of the cell-surface peptidase as a carboxypeptidase-like activity.

Carboxypeptidase-like activity on the surface of CPA47 cells was further suggested based on the inhibition of radiolabeled ANP degradation by a carboxypeptidase inhibitor. Carboxypeptidases are exopeptidases that can be classified as tricarboxypeptidases, dicarboxypeptidases, or carboxypeptidases depending on the number of amino acids released from the C-terminus of a peptide or protein. Other investigators have previously reported the possibility of carboxy-terminal degradation of ANP by cultured cells and by enzymes isolated from tissue [21,26–28]. When examining ANP degradation by bovine vascular smooth muscle cells (BVSMC) at 37°C, Johnson et al. [26] observed that excess unlabeled ANP could not completely inhibit the generation of $^{125}I\text{-}tyr^{28}$ and concluded that a carboxypeptidase might be present on these cells. No further evidence was provided to characterize the postulated carboxypeptidase [26]. However, another group examined ANP degradation in the same cell type (BVSMC) at low temperature (4°C) to inhibit receptor-mediated and fluid-phase endocytosis [7]. Under these conditions, no ANP degradation was observed, suggesting that the enzyme(s) responsible for ANP degradation in BVSMC was not present on the cell surface [7]. Thus, the carboxypeptidase reported by Johnson et al. [26] would likely be an intracellular peptidase in BVSMC, and not the cell-surface peptidase demonstrated in these studies.

Investigators have also examined metalloenzymes secreted by endothelial and neuroblastoma cells that cleave a C-terminal tripeptide from ANP [21,27]. We have also observed that CPA47 cells secrete a similar metalloenzyme [11]. However, the surface peptidase(s) on CPA47 cells was active in the presence of EDTA (Fig. 2). Hence, the cell-secreted metalloenzyme is unlikely to account for the divalent cation-independent ANP degradation observed on the surface of CPA47 cells.

Another C-terminal peptidase, atrial dipeptidyl carboxyhydrolase, has been described as degrading atriopeptins II and III [28]. This enzyme also is unlikely to account for the divalent cation-independent surface degradation of ANP on CPA47 cells since atrial dipeptidyl carboxyhy-

drolase is a metalloprotease isolated from the microsomal fraction enriched with atrial granules. Thus, the characteristics of ANP degradation by the surface of CPA47 cells are unique for a carboxypeptidase-like activity and suggest that the surface peptidase(s) on CPA47 cells is a newly characterized enzyme(s) involved in the degradation of ANP.

Based on our results, it is unlikely that the surface peptidase(s) on CPA47 cells is NEP 24.11 or kallikrein, both of which are known to degrade ANP in vivo [4,5,20]. NEP 24.11 is a metalloprotease, the activity of which is inhibited by EDTA and phosphoramidon and not inhibited by carboxypeptidase inhibitor [20]. Furthermore, bradykinin, the preferred substrate for NEP 24.11 [25], was not effective at inhibiting ANP degradation by this cell-surface peptidase(s) (Table II). These data demonstrate that the cell-surface peptidase(s) is not NEP 24.11. Kallikrein is also unlikely to be responsible for ANP degradation on CPA47 cells, since ANP degradation by kallikrein is inhibited by aprotinin with an IC_{50} of 2.2 μg/ml [8], and ANP degradation by CPA47 cells was not significantly affected by an aprotinin concentration of 10 μg/ml.

The broad, near-physiologic pH range for ANP degradation and the relatively high K_m of the cell-surface ANP peptidase(s) compared to physiological levels of ANP (3.3–23.3 pM [29]) would be characteristic of an enzyme involved in the clearance and degradation of hormones and other molecules from the bloodstream. An excellent example of such an enzyme is NEP 24.11. This enzyme, known to be involved in the clearance and degradation of ANP in vivo [8], has a K_m for ANP degradation of ~10 μM in kidney membranes, which is five orders of magnitude higher than the physiologic concentration of ANP [20]. When compared to endoprotease 24.11, CPA47 cell-surface ANP peptidase(s) has an approximately twentyfold lower apparent K_m suggesting that the CPA47 cell-surface peptidase(s) has a higher affinity for ANP than NEP 24.11 in kidney membranes. These data suggest that the surface enzyme(s) on CPA47 cells could degrade ANP in vivo.

The importance of a carboxypeptidase in the degradation of ANP in vivo has been noted by Krieter and coworkers [18], who suggested that a carboxypeptidase participated in the initial metabolism of ANP in vivo since ^{125}I-tyr was recovered in the plasma 30 sec after administration of ^{125}I-ANP (103–126) in rats. The presence of a surface carboxypeptidase on endothelial cells would explain this result [18]. No internalization would be necessary and the ANP in the plasma would come in immediate contact with endothelial cells. Other well-characterized mechanisms for ANP degradation, receptor-mediated endocytosis and NEP 24.11, are unlikely to account for the rapid release of ^{125}I-tyr. Receptor-mediated endocytosis of ANP would not account for the release of ^{125}I-tyr, since internalization, degradation, and release of degradation products by receptor-mediated endocytosis normally takes longer than 30 sec [30]. Furthermore, ^{125}I-tyr is not a major degradation product of the ectoenzyme NEP 24.11 found in kidney membranes [20]. Hence, an ANP surface carboxypeptidase-like activity on endothelial cells may quickly degrade or modify ANP, thereby affecting its physiologic response further downstream. Thus, an important mechanism for ANP catabolism and regulation in vivo could involve the peptidase(s) activity described here.

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